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Reevaluation of chloride's regulation of hemoglobin oxygen uptake: The neglected contribution of protein hydration in allosterism

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ABSTRACT **We have measured hemoglobin oxygen uptake vs. the partial pressure of oxygen, with independently controlled activities of chloride and water. This control is effected by combining different concentrations of NaCl and sucrose in the bathing solution to achieve: (*i*) water activities were varied and Cl^- activity was fixed, (*ii*) both water and Cl^- activities were varied with a traditional NaCl titration, or (*iii*) Cl^- activities were varied and water activity was fixed by adding compensating sucrose. Within this analysis, the Cl^--regulated loading of four oxygens can be described by the reaction $Hb{\cdot}Cl^- + 4\ O_2 + 65\ H_2O \rightleftharpoons Hb{\cdot}4O_2{\cdot}65H_2O + Cl^-$. The dissociation of a neatly integral chloride, rather than the nonintegral 1.6 chlorides inferred earlier from simple salt titration, demonstrates the need to recognize the potentially large contribution from changes in water activity when titrating weakly binding solutes. The single-chloride result might simplify structural considerations of the action of Cl^- in hemoglobin regulation.**

Among the "allosteric effectors" regulating hemoglobin oxygen affinity, Cl^- has long been known to have a significant physiological role. From *in vitro* studies of the relation between O_2 binding and Cl^- activity, it has been thought that some 1.6 chlorides bind to stabilize the deoxy or T form (1–4), although this stoichiometry is puzzling when one attempts to locate it on the x-ray structure (4, 5). We recently reported (6) that the oxygen affinity of hemoglobin correlates with water activity in the presence of several neutral solutes. We inferred a change of some 60–65 waters in protein hydration when hemoglobin goes from the deoxy T state to the fully oxygenated R state. The water activity in physiological salt solutions is comparable to the water activities used with the neutral solutes that showed the contribution of protein hydration. Therefore, we now have reexamined the regulatory action of chloride, explicitly including changes in protein hydration and the dependence of water activity on added salt. We now find that an alternate description of the data has only a single allosteric Cl^- ($\Delta n_{Cl^-} = -1.02 \pm 0.02$) directly linked with oxygenation during the deoxy-to-oxy transition, while some 65.2 ± 2.4 additional water molecules bind to the protein.

Most, perhaps all, weakly binding effectors such as chloride necessarily work at high enough concentrations that their presence also significantly changes the activity of water. Ligand regulation of hemoglobin and other allosteric proteins should be reevaluated with regard for the activity of water and molecular hydration, as first anticipated by Tanford (7).

MATERIALS AND METHODS

Freshly drawn human blood was washed three times in isotonic salt solutions and the erythrocytes were then lysed with distilled water. Debris was removed by centrifugation (Sorvall RC-5B, 27,000 × *g* for 1 h at 4°C). The resulting hemoglobin solution supernatants were then eluted over a PD 10 Sephadex G-25M (Pharmacia) column equilibrated with 0.1 M NaCl/0.05 M Tris·HCl, pH 7.5. The protein solution was next passed over an AG-501-X8 deionization column (Bio-Rad). Final hemoglobin solutions were concentrated by centrifugation with a Centricon-30 microconcentrator and buffered to pH 7.0 with 0.05 M Bis-Tris·acetic acid.

Hemoglobin oxygen binding curves were measured at 25°C by using a Hem-O-Scan oxygen dissociation analyzer (SLM Aminco, Rochester, NY) as described (6). Hemoglobin samples were deoxygenated with a 5.6% CO_2/94.4% N_2 gas mixture and subsequently oxygenated with a 25% O_2/5.6% CO_2/69.4% N_2 mixture. The oxygen partial pressures at hemoglobin half-saturation (P50 values) were determined from standard Hill plots.

Hemoglobin samples at fixed water activity and various Cl^- activities (a_w-constant) were prepared combining NaCl and an appropriate amount of sucrose (Ultrapure Grade, BRL) at each NaCl concentration. Sample osmolalities were measured with a vapor pressure osmometer (Wescor model 5100C, Logan, UT). When NaCl was titrated and water activity was allowed to vary with salt concentration (a_w-free), no sucrose was added.

THERMODYNAMIC ANALYSIS

The number of Cl^- ions allosterically coupled to hemoglobin oxygenation is typically extracted from the sensitivity of the P50 value to Cl^- concentration by applying a standard Wyman linkage relation (7–9),

$$\frac{d(\ln P50)}{d(\ln[Cl^-])} = \frac{\Delta n_{Cl^-}}{\Delta n_{O_2}}. \quad [1]$$

The slope of ln P50 vs. ln[Cl^-] gives the change in the number of Cl^- ions bound to the hemoglobin tetramer, Δn_{Cl^-}, linked to the binding of four oxygens, Δn_{O_2}, and to the transition from the deoxy-T to the oxy-R state. This straightforward interpretation assumes that the activities of all other "allosteric" solution components are held constant.

Changing the activity of one solution component, however, also changes the activity of water. Previously, we used this coupling of solute and water activities to show that water itself can be considered an allosteric ligand (6). We extracted an apparent change in hemoglobin water binding associated with oxygenation from the sensitivity of P50 to water activity. The Wyman linkage relation coupling oxygen uptake and water activity is homologous to Eq. **1**,

$$\frac{d(\ln P50)}{d(\ln a_w)} = \frac{\Delta n_w}{\Delta n_{O_2}}. \quad [2]$$

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In that earlier work, we used four neutral solutes (sucrose, stachyose, triethylene glycol, and 400-Da polyethylene glycol) as osmotic agents. We concluded that their action was better described as a function of the activity of water rather than as a function of direct solute–hemoglobin interaction (though both possibilities were quantitatively evaluated). Since the effect scaled as a function of water activity rather than solute type or concentration, it is also unlikely to be due to solute-specific changes in solution dielectric constant, as suggested by Bellelli *et al.* (10). On the strength of that previous analysis, therefore, we use the convention that there is no allosteric effect from direct binding of these solutes to hemoglobin.

Osmotic stress effects rely on an exclusion of solutes from waters hydrating macromolecules, either due to steric interactions (solutes are simply too large to penetrate water-filled cavities) or to repulsive chemical interactions with exposed surfaces [as reflected in the preferential interaction coefficients introduced by Timasheff (11)]. It is essential in osmotic stress studies to work with solutes not only of different sizes but also of different chemical natures to distinguish solute-independent osmotic effects from solute-specific binding and from solute-dependent exclusion from specific exposed surfaces.

Two methods can be used for now reevaluating the effect of Cl^- on hemoglobin oxygen binding that explicitly consider not only the allosteric action of direct Cl^- binding but also the effect of salt on water activity and the consequent allosteric action of water. In the a_w-constant method, as the Cl^- concentration is changed, sufficient sucrose is added to keep the water activity constant, (equaling that of the highest salt concentration used, 0.8 M NaCl). In this case, Eq. **1** is strictly applicable. Alternatively, in the a_w-free case, salt is added but the water chemical potential is not clamped by compensating sucrose. The data can be analyzed for changes in both Cl^- and water binding, however, by applying Gibbs–Duhem relations.

Changes in the chemical potential of hemoglobin (μ_{Hb}) can be related to changes in O_2, Cl^-, and water chemical potentials, μ_{O_2}, μ_{Cl^-}, and μ_w, respectively, through the number of each allosteric species, n_{O_2}, n_{Cl^-}, and n_w, associated with the protein

$$-d\mu_{Hb} = +n_{O_2}d\mu_{O_2} + n_{Cl^-}d\mu_{Cl^-} + n_w d\mu_w. \quad [3]$$

The chemical potentials of oxygen and Cl^- are given by

$$d\mu_{O_2} = RT\, d(\ln Po_2)$$

$$d\mu_{Cl^-} = RT\, d(\ln\, \gamma[\mathrm{NaCl}]) = RT\left\{1 + \frac{d(\ln\,\gamma)}{d(\ln[\mathrm{NaCl}])}\right\} d(\ln[\mathrm{NaCl}]). \quad [4]$$

We assume that both Na^+ and Cl^- contribute equally to the activity coefficient, γ, of NaCl. Over the range of salt concentrations we examine, $d(\ln\,\gamma)/d(\ln[\mathrm{NaCl}])$ is almost constant (12), with $\Gamma = \{1 + d(\ln\,\gamma)/d(\ln[\mathrm{NaCl}])\} = 0.937$ for molar salt concentration units.

Salt and water chemical potentials in the bulk solution are not independent but are coupled by another Gibbs–Duhem relation,

$$d\mu_w = -\left(\frac{x_{NaCl}}{x_w}\right) d\mu_{NaCl} = -RT\left(\frac{2\Gamma[\mathrm{NaCl}]}{55.5}\right) d(\ln[\mathrm{NaCl}]), \quad [5]$$

where x_{NaCl} and x_w are the mole fractions of salt and water, respectively. The protein and oxygen contributions to $d\mu_w$ have been omitted since x_{O_2}, $x_{Hb} \ll x_{NaCl} \ll x_w$.

Incorporating Eqs. **4** and **5** into Eq. **3**, we have

$$-d\mu_{Hb} = RTn_{O_2}d(\ln Po_2) + RT(\Gamma n_{Cl^-} - \Phi n_w[\mathrm{NaCl}])d(\ln[\mathrm{NaCl}]), \quad [6]$$

where $\Phi = 2\Gamma/55.5 \approx 0.033$.

The change in bound oxygen with salt concentration is described by a Maxwell crossrelation,

$$\left.\frac{\partial n_{O_2}}{\partial(\ln[\mathrm{NaCl}])}\right|_{\ln Po_2} = \left.\frac{\partial(\Gamma n_{Cl^-} - \Phi n_w[\mathrm{NaCl}])}{\partial(\ln Po_2)}\right|_{\ln[\mathrm{NaCl}]}. \quad [7]$$

In practice, then, the observed sensitivity of oxygen binding to the solution NaCl concentration can be converted into the change in both chloride and water binding during oxygenation at constant salt activity. Transforming this equation into a standard linkage relation (8, 9) gives

$$\frac{d(\ln P50)}{d(\ln[\mathrm{NaCl}])} = \Gamma\frac{\Delta n_{Cl^-}}{\Delta n_{O_2}} - \Phi\frac{\Delta n_w[\mathrm{NaCl}]}{\Delta n_{O_2}}, \quad [8]$$

where P50 is taken as the median ligand activity, p_m, defined in refs. 8 and 9. This equation, describing the coupling of both direct solute binding and osmotic effects, is analogous to one derived by Tanford (7) and applied by Haire and Hedlund (13) to the dependence of hemoglobin O_2 affinity on ethylene glycol concentration. Both $\Delta n_{Cl^-}(n_{Cl}^{oxy} - n_{Cl}^{deoxy})$ and $\Delta n_w(n_w^{oxy} - n_w^{deoxy})$ contribute to the slope $d(\ln P50)/d(\ln[\mathrm{NaCl}])$. By definition, Δn_{Cl^-} and Δn_w are changes in the numbers of bound Cl^- ions and water molecules associated with binding of four oxygens to hemoglobin, stabilizing one or the other form of the protein, and therefore, regulating oxygenation.

RESULTS

The change of ln P50 with added salt is shown in Fig. 1 for the two cases: with water activity held constant and with water activity allowed to change with salt concentration. With fixed water activity (a_w-constant), the linear dependence of ln P50 on Cl^- chemical potential implies a constant Δn_{Cl^-} over the salt concentration range examined. The slope, $d(\ln P50)/d(\ln[\mathrm{NaCl}])$, corresponds to the release of 1.06 ± 0.06 chlorides per hemoglobin tetramer in the transition from fully deoxygenated to fully oxygenated states. When the same equation is applied to the data with water activity allowed to vary with added salt (a_w-free), the average slope over the salt concentration range 0.05–0.8 M gives the previously inferred 1.6 chlorides per hemoglobin (1–4). The curvature in the plot has generally been interpreted to indicate that there are at least two allosteric chloride binding sites, one of which binds Cl^- only weakly.

The difference between the a_w-constant and the a_w-free Cl^- titration results reflects the potentially large contribution to the deoxy-T ⇌ oxy-R transition from differences in the number of solute-excluding water molecules solvating the two states. If, in addition to differences in the number of Cl^- ions directly bound to the T and R states, salt also acts "osmotically" as do the neutral solutes investigated previously (6) so that salt too is excluded from hemoglobin-solvating water, then the a_w-constant experiment is the proper one for extracting the allosteric effect of direct Cl^- binding only. The a_w-free experiment would in this case probe not only changes in Cl^- binding but also changes in hemoglobin hydration, a situation corresponding to Eq. **8.**

The best two-parameter fit of Eq. **8** to the a_w-free Cl^- titration data of Fig. 1 is shown in Fig. 2. The adjustable parameters Δn_{Cl^-} and Δn_w are assumed to be independent of chloride concentration. We find the curve is best described by $\Delta n_{Cl^-} = -1.02 \pm 0.02$ and $\Delta n_w = +65 \pm 2.4$. The nonlinearity of the data is now seen to be a natural conse-

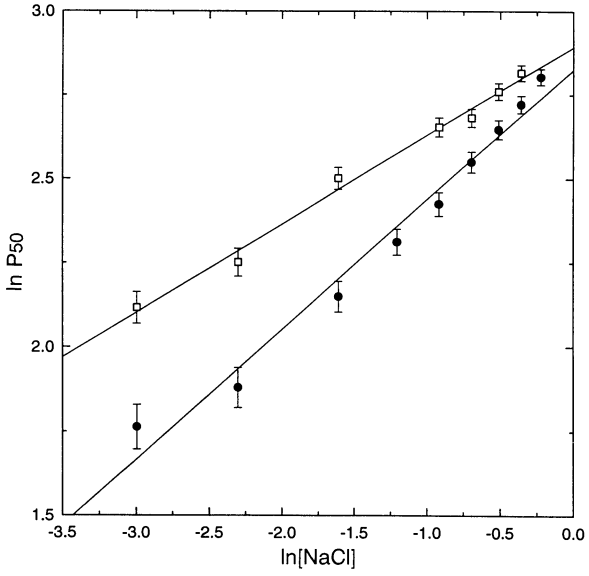

FIG. 1. Effect of chloride concentration on the oxygen partial pressure at half saturation of hemoglobin, P_{50}, is shown for two cases: a simple salt titration without controlling water activity and a salt titration under conditions of fixed water activity (●). Measurements were made in the usual way with water activity allowed to change freely with added salt. The best fitting straight line to the data gives an apparent release of 1.6 Cl^- per hemoglobin tetramer during the deoxy-to-oxy transition, in accord with earlier reports (1–4) (□). The activity of water was maintained at 1.50 ± 0.05 osmoles/kg of water by combining NaCl with an appropriate amount of sucrose. Osmotic pressures of sucrose and NaCl were found to be additive to within about 5%. The data points shown are the average of three sets of measurements. The slope here gives a release of only 1.06 ± 0.06 chloride ions associated with the fully deoxy T to fully oxy R state transition. Data sets at other constant water activities ranging from 1.0 to 1.5 osmoles/kg of water gave identical results.

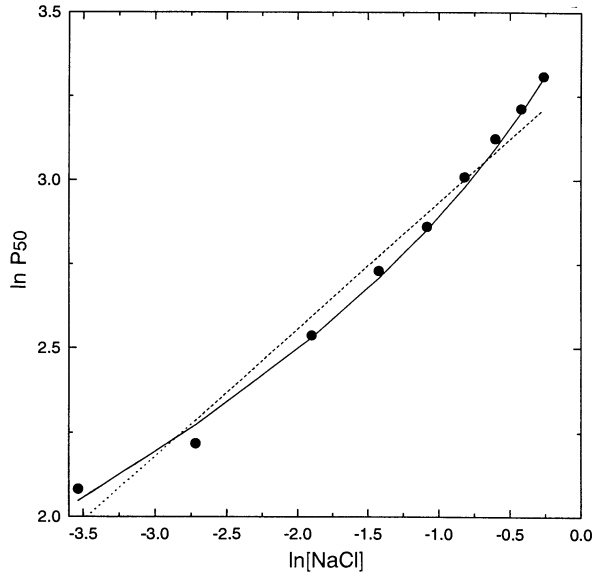

FIG. 2. Dependence of hemoglobin oxygenation to chloride concentration with water activity allowed to vary with salt concentration is well described by recognizing a contribution from changes in water binding. Each point is the average of four chloride titration experiments. The dotted line is the linear fit to the data used with Eq. **1** for calculating Δn_{Cl^-} neglecting the contribution from changes in water activity with added salt. The solid line is a nonlinear fit to the data using the integrated form of Eq. **8** that recognizes the concomitant change in water activity with chloride activity. The best fit to the data gives the changes in both Cl^- and water binding associated with the fully deoxy to fully oxy form transition. The change in bound chloride, $\Delta n_{Cl^-} = -1.02 \pm 0.02$ Cl^- per hemoglobin tetramer, agrees with that measured under conditions of constant water activity (shown in Fig. 1). The concomitant change in bound water is calculated as $\Delta n_w = +65.2 \pm 2.4$ H_2O molecules per hemoglobin tetramer, agreeing well with the hydration change measured by the application of neutral solutes (6).

quence of the nonlinear dependence of water chemical potential on ln[NaCl].

DISCUSSION

By recognizing changes in water activity, we are able to generate self-consistent results for changes in the numbers of Cl^- ions (Δn_{Cl^-}) and water molecules (Δn_w) during the deoxy-T ⇌ oxy-R transition. From preparations with fixed Cl^- activity and varied water activity, we previously found $\Delta n_w = +60$ to 65 molecules of water per hemoglobin tetramer. Now, with water activity fixed and Cl^- activity varied, we find $\Delta n_{Cl^-} = -1$ (Fig. 1). A separate nonlinear fit to data with both Cl^- and water activities allowed to vary closes the circle with $\Delta n_{Cl^-} = -1$ and $\Delta n_w = +65$ per tetramer (Fig. 2). By recognizing the importance of water activity, one sees that about 40% of the previously inferred $\Delta n_{Cl^-} = -1.6$ could actually reflect the association of an additional 65 waters. The energetics of binding this extra water with oxygenation depends on Cl^- concentration only indirectly, through the effect of added salt on water activity.

The changes in chloride and water binding we infer are differences in the association of these ligands to particular hemoglobin conformations. Given the sensitivity of protein structure and dynamics, in general, to ligand binding, we must emphasize that these results are strictly applicable only to the particular range of solution conditions (pH, NaCl concentration, CO_2 partial pressure, water activities, temperature, etc.) and hemoglobin species examined here. The method used to prepare the hemoglobin, for example, does not remove all organic phosphates. It has previously been observed that the chloride dependence of O_2 affinity is sensitive to the binding of organic phosphates to hemoglobin (14). The osmotic sensitivity of O_2 binding might well also depend on the binding of organic phosphates.

It is conceivable that the osmotic equivalence of NaCl and neutral solutes is only a coincidence and that a different 65 water molecules exclude salt and sugar. It might even be that chloride is not acting osmotically at all, that the traditional weak Cl^- binding picture still holds, and that the constant-water-activity result is simply an additive effect of sucrose. It is possible that the apparent single extra bound chloride is really the aggregate of fractional chlorides at different mutually excluding sites that happen to add up to one or that it is reflecting differences in proximity of neutralizing chloride ions to positive hemoglobin charges in the central cavity in the two forms (4).

Nevertheless, there is now a pleasing possibility that the correlation of Hb oxygenation and hydration extends beyond the neutral solutes used previously and that many solutes as salt act osmotically on the T ⇌ R transition. If this is true, then one must now reexamine the action of all weakly binding effectors to gauge the extent of their own activity rather than that of solvent water still so neglected in protein regulation.

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